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Early antibiotic selection and efficient rooting and acclimatization improve the production of transgenic plum plants (*Prunus domestica* L.)

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Abstract We describe here an improved system for routinely developing transgenic plum plants (*Prunus domestica* L.) through the use of *Agrobacterium tumefaciens*. The production of non-transformed "escapes" has been virtually eliminated, and rates of plant establishment in the greenhouse have been dramatically improved. The system is based on the regeneration of shoots from hypocotyls extracted from mature seed. The shoot regeneration medium is Murashige and Skoog (MS) salts and vitamins supplemented with 7.5 μM thidiazuron and 0.25 μM indole-butyric acid. Transferring the explants after co-cultivation to shoot regeneration medium containing 80 mg l^{-1} of kanamycin and 300 mg l^{-1} of Timentin reduced the total number of regenerated shoots without affecting the transformation rate. Transformation rates using the described system averaged 1.2% of the hypocotyl slices producing transgenic plants, with a range of 0–4.2%. The transgenic shoots rooted at a rate of 90% on half-strength MS salts and vitamins supplemented with 5 μM α-naphthaleneacetic acid and 0.01 μM kinetin. Plantlets were transferred to a greenhouse directly from culture tubes with a 90% average survival.

Keywords Antibiotic selection · Rooting · Viral coat protein

Abbreviations *ACO1: Prunus persica* 1-aminocyclopropane-1-carboxylate oxidase · *CP:* Coat protein · *GUS:* β-Glucuronidase · *NPTII:* Neomycin phosphotransferase II · *PCR:* Polymerase chain reaction · *PDV:* Prune dwarf virus · *PNRSV: Prunus* necrotic ringspot virus · *SGM:* Shoot growth medium · *SRM:* Shoot regeneration medium · *TIM:* Timentin (SmithKline Beecham, Philadelphia) · *TomRSV:* Tomato ringspot virus

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Introduction

Since our initial report of hypocotyl transformation of plum (*Prunus domestica* L.) (Mante et al. 1991), we have successfully used this technology to transfer various transgenes, including the NPTII and GUS marker genes, and viral CP genes into this species (Scorza et al. 1994, 1995a, 1995b). This method utilizes MS medium (Murashige and Skoog 1962) supplemented with thidiazuron (TDZ) and indole-3-butyric acid (IBA) in ratios ranging from 2:1 to 5:1. While the technique consistently produces transgenic plum plants, there have been several areas in need of improvement. Repeated transfers are required to eliminate non-transformed shoots that survive for long periods under selection ("escapes"), and a relatively low percentage of transgenic shoots root (25–50%) and survive in the greenhouse. We report here several significant modifications of previous work (Mante et al. 1989, 1991) that improve the efficiency and reliability of the system. We have developed an antibiotic selection protocol that virtually eliminates the production of non-transformed escapes. The elimination of multiple transfers that are generally required to identify non-transformed escapes saves time, labor and the expense of consumables. We have also changed both the rooting and the acclimatization methods to dramatically increase the rooting percentage and to reduce acclimatization time while maintaining a high survival rate and good plant development in the greenhouse. These protocols improve the utility of plum as a model stone fruit species for genetic manipulation.

Materials and methods

Agrobacterium preparation

Agrobacterium tumefaciens strains EHA105 (Hood et al. 1993) and LBA4404 (Hoekema et al. 1983) carrying plasmid pBISN1 (Narashimhulu et al. 1996), pGA482GG (An et al. 1985) or pGA428GGi (a modification of the plasmid pGA482GG that includes changes in the multiple cloning site and the addition of an

Table 1 *Agrobacterium* strains, vectors, selection and marker genes used for plum (*Prunus domestica* L.) transformation

Strain	Plasmid/ gene	Selection	Marker gene
EHA105	BISNI	Kan^{50} Rif^{20}	NPTII, GUS
EHA105	BISNI/PDV-CP	Kan^{50} Rif^{20}	NPTII
EHA105	BISNI/PNRSV-CP	Kan^{50} Rif^{20}	NPTII
LBA4404	BISNI/PDV-CP	Kan^{50} Rif^{20}	NPTII
LBA4404	BISNI/PNRSV-CP	Kan^{50} Rif^{20}	NPTII
EHA105	GA482GG/TomRSV-CP	Rif^{25} Gen^{50}	NPTII, GUS
LBA4404	GA482GG/TomRSV-CP	Rif^{25} Gen^{50}	NPTII, GUS
EHA105	GA482GGi/AntisenseACO1	Rif^{20} Gen^{50}	NPTII, GUS

intron in the GUS gene to provide for plant-specific expression) were used as vector systems for transformation. These plasmids contained the reporter and selectable marker genes GUS and NPTII (Table 1). The PNRSV and PDV CP genes were engineered into the plasmid pBISN1 by replacing the GUS gene with the CP gene and were kindly provided by R. Hammond, USDA-ARS, Beltsville, Maryland. The TomRSV CP gene (kindly provided by D. Gonsalves, USDA-ARS Hilo, Hawaii) and the antisense ACO1 gene (kindly provided by A.M. Callahan, USDA-ARS, Kearneysville, West Virginia) were engineered into the plasmid pGA482GG and pGA428GGi, respectively, without removing the GUS gene. The resulting plasmids were designated as pBISNI/PNRSV-CP, pBISNI/PDV-CP, pGA482GG/TomRSV-CP and pGA428GGi/antisenseACO1. The strain EHA105 carrying the original plasmid pBISN1 was used to produce transformed controls with marker genes.

Engineered *Agrobacterium* strains were grown on Luria-Bertani medium (10 g l^{-1} Bacto-tryptone, 5 g l^{-1} Bacto-yeast extract, 1 g l^{-1} NaCl, 1 g l^{-1} glucose; pH 7.0) solidified with 15 g·l^{-1} Bacto-agar (Bacto-products, Difco Laboratories, Detroit, Mich.), with appropriate antibiotics (Table 1). For co-cultivation, a single colony was inoculated into 10 ml of Luria-Bertani medium with appropriate antibiotics, incubated for 5 h with constant agitation at 175 rpm, at which time an additional 40 ml was added and grown overnight at 28°C with constant agitation (175 rpm). Overnight culture growth had an O.D. at 600 nm of between 0.2 and 1.0. Cultures were centrifuged at 5,000 *g* for 10 min and resuspended in 50 ml of bacterial resuspension medium consisting of MS salts, 2% (w/v) sucrose, 100 μM acetosyringone and 1 m*M* betaine phosphate (James et al. 1993). Cultures in bacterial resuspension medium were shaken (175 rpm) at 25°C for 5 h before use.

Plant culture media and culture conditions

The SRM consisted of 3/4-strength MS salts supplemented with (in mg l^{-1}) myo-inositol, 100; thiamine HCl, 0.1; nicotinic acid, 0.5; pyridoxine HCl, 0.5; glycine 2.0; sucrose 2% (w/v); agar (Ultrapure, USB, Cleveland, Ohio, or similar) 0.7% (w/v). The growth regulators used to induce shoot regeneration from hypocotyl slices were 7.5 μM TDZ and 0.25 μM IBA. The medium was adjusted to pH 5.8 using 1 *N* KOH and autoclaved at 1.4 kg cm^{-2}. TDZ and the antibiotics were filter-sterilized and added to autoclaved medium when required. The SGM comprised basal SRM with TDZ replaced by 1–3 μM benzylaminopurine (BAP). The basal rooting medium was 1/2-strength MS medium with SRM organics and 0.01 μM K and 0.01 μM α-naphthaleneacetic acid (NAA). SRM was dispensed into 100×20-mm petri plates (25 ml each), SGM into petri plates (25 ml each) or Magenta vessels (Magenta, Chicago, Ill.) (50 ml/vessel) and rooting medium into culture tubes (15 ml/tube).

The cultures were grown in the culture room at 24±1°C under a 16/8-h (light/dark) photoperiod with light provided by an equal mixture of warm-white fluorescent and Vita-lite full-spectrum fluorescent (Duro-test, Bergen, N.J.) lamps at an intensity of 45–50 μE m^{-2} s^{-1}.

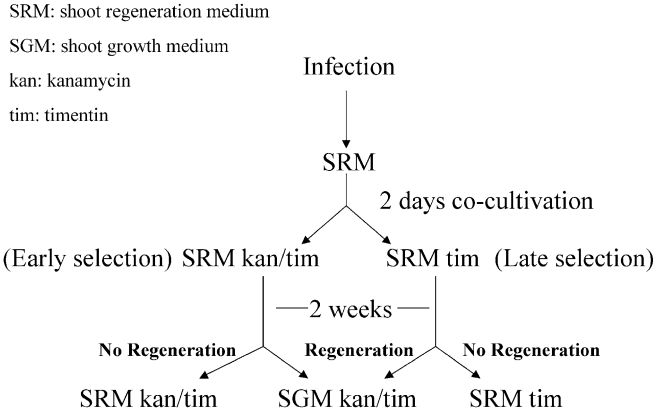

Fig. 1 Plum (*Prunus domestica* L.) regeneration and transformation scheme

Explant preparation

Following the collection of mature fruit, the mesocarp (flesh) was removed from the endocarp (stone), which, with enclosed seed, was cleaned and allowed to air dry 2–3 days at room temperature. Seeds were stored at 4°C in plastic mesh bags. 'Stanley' and 'Bluebyrd' plum seeds that had been stored for 1 year and seeds from the current season harvest were used. After the endocarp had been removed with a nutcracker, the seeds were surface-disinfected by immersion in a 1% sodium hypochlorite solution containing approximately 20 μl Tween-20 per 100 ml solution for 30 min and rinsed three times (5 min each time) with sterile distilled water in a laminar flow bench. Disinfected seeds were soaked in sterile water overnight at room temperature and the seed coats removed with the aid of a scalpel. The radicle and the epicotyl were discarded, and the hypocotyl was sliced into three cross sections (0.5–1 mm), which were used for regeneration/transformation.

Transformation/regeneration

Hypocotyl slices were used on the same day that they were cut. Slices were immersed in resuspended *Agrobacterium* for about 20 min, blotted briefly on sterile filter paper and placed on co-cultivation medium (SRM without antibiotics). After 2 days the explants were washed in a sterile solution of 1/2-strength MS medium with 300 mg l^{-1} TIM and blotted briefly on sterile filter paper. Half of the explants were placed on SRM with 300 mg l^{-1} TIM (late selection) and the other half on SRM with 80 mg l^{-1} kanamycin (kan) and 300 mg l^{-1} TIM (early selection). The explants regenerating shoots cultured on SRM with only TIM were transferred to a medium containing kan after 2 weeks (Fig. 1).

The transformation experiments were repeated four times for each *Agrobacterium*/vector combination. For the vectors containing the PDV-CP, PNRSV-CP and the TomRSV-CP, 50 seeds were used (approximately 150 hypocotyl slices). From these, 60 slices were explanted on to SRM-300 mg l^{-1} TIM after co-cultivation, 60 were

explanted on to SRM-80 mg l^{-1} kan/300 mg l^{-1} TIM and 30 slices were used as controls and cultivated, without infection, on SRM without antibiotics. In the experiments using the vector pGA482G-Gi/antisenseACO1, only late selection was used. After 14 days, shoots began to appear, mainly in late selection, and explants with shoots were transferred to SGM. Explants without shoots were transferred to fresh medium without kan. In the late selection treatment, explants were exposed to kan following the appearance of shoots. Explants were subcultured every 3 weeks in petri plates. When the regenerated shoots were longer than 1 cm they were separated from the hypocotyl slice and cultured onto SGM in Magenta vessels and subcultured every 4 weeks.

Confirmation of transformation

Putative transformants, identified by growth on kan-containing medium, were assayed using three methods to verify gene incorporation and expression. Initially, the plants were evaluated for GUS expression using the X-glucuronide histochemical assay (Jefferson 1987). A blue staining was often visible in 2 h or less, even though tissues were incubated in the substrate solution overnight at 37°C. After this incubation period, the tissues were cleared with 95% ethanol to more easily visualize the blue staining. Two other methods for evaluation, PCR and DNA blotting (Southern 1975), required the isolation of high-quality genomic DNA from putative transformants. We used a modified CTAB DNA extraction protocol as previously described (Scorza et al. 2001). PCR analyses of putative transformants were carried out using the NOS/NPTII and GUS primers (Scorza et al. 1994, 1995b). The primers used for the PNRSV-CP and PDV-CP genes were obtained from R. Hammond; those used for the TomRSV-CP gene insert were those described by Scorza et al. (1996). PCR reactions were run using the GenAmp kit components (Perkin-Elmer, Norwalk, Conn.) with occasional minor variations in the following cycle parameters: 1 min at 94°C, 1.5 min at 55°C (65°C for GUS) and 2 min at 72 °C. The first cycle used an additional 3-min melt at 95°C and the last five cycles had 4-min extension times at 72°C. After 35 amplification cycles, the PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

DNA blotting

DNA was quantified spectrophotometrically, digested with appropriate restriction enzyme(s), resolved on an agarose gel and transferred to a positively charged nylon membrane (Sambrook et al. 1989). DNA blots were non-radioactively probed (Scorza et al. 2001). The primers and probes for DNA blotting were generated by PCR using Digoxigenin dUTP in the reaction mix as described by the manufacturer (Roche Applied Science, Indianapolis, Ind.).

Rooting

Clumps of axillary shoots which were cultured in Magenta boxes on SGM medium with or without antibiotics (transgenic and non-transgenic control shoots, respectively) were used for the rooting trials. Apical shoots were 1.5–2 cm long with two to three developed leaves. In the initial rooting trials we used a modification of the rooting method described by Scorza et al. (1994). Shoots were dipped in a solution of NAA for 20 s prior to being transferred onto rooting medium in test tubes. In order to improve rooting and to eliminate the auxin dip procedure we studied the effect of increasing the NAA concentration in the medium from 0.01 μM to 5 μM for both control and transgenic shoots and the effect of reducing the kan concentration from 80 mg l^{-1} to 40 mg l^{-1} for the transgenic shoots. After 4 weeks in rooting medium we recorded the percentage of rooted shoots and the number and length of roots from each shoot.

Acclimatization

To evaluate the effect of shoot size and the number and length of the roots on acclimatization success, we used transgenic and untransformed plantlets from rooting experiments. We classified shoots as small (shorter than 1 cm), medium (1–2 cm) or large (more than 2 cm); the numbers of roots as few (1 or 2 roots) or many (2 to 6 roots); root length as short (shorter than or equal to 1 cm), medium (1–4 cm), or long (more than 4 cm). The plantlets were transferred to Magenta vessels containing an autoclaved soil-less potting mixture consisting of Metro-Mix 510 (Scotts-Sierra, Marysville, Ohio):vermiculite:perlite:peat moss at a volumetric ratio of 1:1:0.5:0.25. This medium was moistened with liquid 1/2-strength MS salts, pH 5.8, and the cultures maintained in the tissue culture growth room. After 2 weeks, the lids of the vessels were removed in the laminar flow hood and the plantlets watered as needed with sterile de-ionized water and placed inside resealable plastic bags. The bags were sealed and returned to the culture room. After 1 week, the bags were partially opened, and 1 week later they were fully opened. On the third week, the plantlets were transferred to 3-inch-square peat pots containing the potting mix described above. The plantlets were then sprayed with an aqueous solution (1:20, v/v) of Anti Stress 2000 (Polymer Ag, Fresno, Calif.) and cultured under ambient greenhouse conditions. One month later, the survival percentage was recorded. The experiment was repeated twice with 20 plantlets each.

In a second experiment, we evaluated the transfer of plantlets to the greenhouse directly from tissue culture tubes. Since preliminary observations indicated that shoot size was important for greenhouse acclimatization and survival, we tested shoots ranging in length from 0.9 cm to 4 cm. Shoot height was recorded at the beginning and at the end of the experiment. The experiment was repeated twice with 24 plantlets each. We transferred one-half of the plantlets to 3-inch-square peat pots, sprayed them with the anti-transpirant formulation described above, covered each peat pot with a clean, empty Magenta vessel and placed them in the greenhouse under 50% shade. The other half of the plantlets were transplanted to Magenta vessels containing the sterile potting mix described above and placed in the tissue culture growth room under the light and temperature conditions previously described. Two weeks later these plantlets were transferred to ambient greenhouse conditions, and shoot height and survival were recorded.

Results

Regeneration and selection

Shoot initials developed along the edges of the hypocotyl slices after 2–3 weeks in late selection and after 2–16 weeks in early selection. Irrespective of the orientation of the explants on the surface of the medium, shoots always regenerated from the proximal surface. The percentage of regeneration obtained 7 weeks after co-cultivation and at the end of the experiment, about 28 weeks, is shown in Table 2.

In late selection, the percentage of regeneration ranged between 36.3% and 63.2% and was similar at 7 weeks and 28 weeks. Also in late selection 81.3% of the shoots regenerated in the 2 weeks prior to transfer to kan-containing medium. Once exposed to kan, the non-transgenic shoots began a slow bleaching process, which progressed over several subcultures. About 20% of the explants in late selection produced more than one shoot, and it was difficult to determine if they were from separate transformation events, especially if the shoots

Table 2 Effects of selection method and *Agrobacterium* strain/plasmid/gene combinations on the production of transgenic plum shoots 7 weeks following co-cultivation with *A. tumefaciens* and maximum regeneration at the end of the experiment (approx. 28 weeks). Data are the mean of four repetitions with about 70 hypocotyl sections each (*nt* not tested)

Strain/plasmid/gene	Seven-week regeneration[a] (%)		Maximum regeneration[a] (%)	
	Late selection	Early selection	Late selection	Early selection
EHA105/pBISNI/ GUS	39.2 ab,A	0.73 b,B	39.2 b,A	1.5 c,B
EHA105/pBISNI/ PDVcp	35.4 b,A	1.05 b,B	36.5 b,A	1.8 c,B
LBA4404/pBISNI/ PDVcp	51.8 ab,A	2.73 b,B	54.2 ab,A	4.4 c,B
EHA105/pBISNI/ PNRSVcp	35.6 b,A	2.8 b,B	36.3 b,A	2.8 c,B
LBA4404/pBISNI/PNRSVcp	61.1 a,A	1.7 b,B	63.2 a,A	1.7 c,B
EHA105/pGA482/GG-TomRSVcp	49.9 ab,A	14.5 a,B	49.9 ab,A	17.1 a,B
LBA4404/pGA482/GG-TomRSVcp	56.5 ab,A	9.9 a,B	56.5 ab,A	10.2 b,B
EHA105/pGA482 GGi/antisesnse ACO1	nt	nt	37.5[b]	nt

[a] Means with the same letters in columns (lower case) and in rows (capital letters) are not significantly different at $P<0.5$ according to the Student-Newman-Keuls test
[b] Data from a separate experiment in which early selection was not applied

Table 3 Effects of the selection method and *Agrobacterium* strain/plasmid/gene combinations on the production of transgenic plum shoots 7 weeks following co-cultivation with *A. tumefaciens*, the total number of explants producing transgenic shoots at approximately 28 weeks and the number of transgenic lines at approximately 28 weeks. Data are the mean of four repetitions with about 70 hypocotyl sections each (*nt* not tested)

Strain/plasmid/gene	Number of surviving shoots after 7 weeks		Total number of explants producing transgenic shoots[a]		Total number of lines[b]			
					Putative		Positive (% transformation)	
	Late selection	Early selection	Late selection	Early selection	Late selection	Early selection	Late selection	Early selection
EHA105/pBISNI/GUS	45	0	1	1	1	1	1 (0.4)	1 (0.4)
EHA105/pBISNI/PDVcp	94	8	0	2	0	19	0 (0)	4 (1.4)
LBA4404/pBISNI/ PDVcp	111	6	0	2	0	2	0 (0)	2 (0.4)
EHA105/pBISNI/PNRSVcp	61	5	1	1	1	1	1 (0.4)	1 (0.7)
LBA4404/pBISNI/PNRSVcp	85	2	2	2	2	2	2 (0.7)	2 (0.7)
EHA105/pGA482/ GG-TomRSVcp	66	6	10	3	13	3	11 (3.7)	3 (1.1)
LBA4404/pGA482/ GG-TomRSVcp	74	18	8	3	22	3	12 (4.2)	3(1.1)
EHA105/pGA482GGi/ antisenseACO1	–	–	–	–	–	–	12[c](2)	–

[a] Maximum number of shoots obtained
[b] Total number of lines at the end of the experiment
[c] Data from a separate experiment in which early selection was not applied

were in proximity to each other. We studied the uniqueness of the transformation events using the plasmid pGA482GGi/antisenseACO1 in *A. tumefaciens* strain EHA105. In this study, one explant produced eight shoots, all of which grew well in kan-containing medium. An analysis of the transgene integration patterns through DNA blotting indicated that three of the eight shoots were independent transformants (data not presented). In early selection, most of the hypocotyl slices remained white and unexpanded. The percentage of regeneration was significantly lower than in late selection and ranged between 1.5% and 17.1% (Table 2). Most of the slices produced only one shoot, and some shoots regenerated after several subcultures on kan-containing SRM. The antibiotic TIM included in the selection medium to control the growth of *Agrobacterium* did not inhibit shoot development from the hypocotyl slices on SRM; to the contrary, we observed a significantly higher regeneration percentage in late selection (44.6%) than in the control explants, the latter not inoculated with *Agrobacterium* and cultured in SRM without antibiotics (25.9%).

Selection method

The number of shoots surviving 7 weeks post-co-cultivation was significantly higher in late selection (45–111 shoots) than in early selection (0–18 shoots) (Table 3). However, at the end of the experiment, the number of explants producing transgenic shoots was similar (Table 3) and generally higher for early selection than for late selection with the exception of plasmid pGA482GG-TomRSV-CP. The transformation efficiency (based on the number of transgenic regenerants per inoculated explant × 100) ranged from 0.4% to 4.2% (Table 3). The number of non-transformed "escape" shoots can be

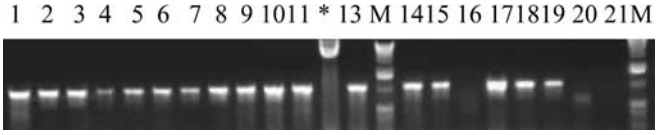

Fig. 2 Amplified 1,100-bp NPTII gene fragment from transgenic plums. *Lines*: *1–11*, *13*, *14*, *15*, *17–19 nptII*-positive plants; *16*, *20* non-transformed controls; *21* no DNA control. *M* 1-kb DNA ladder, * a lane where a 123-bp ladder was loaded but failed to run properly

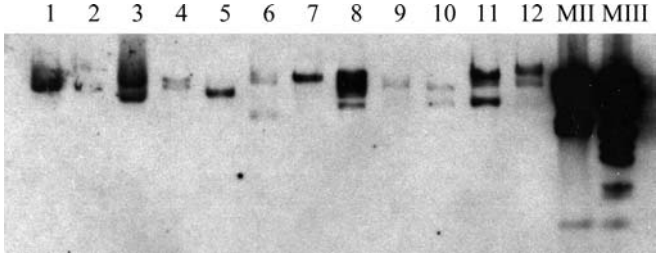

Fig. 3 DNA blot analysis of 12 transgenic plums *Lines 1–12* transformed with the TomRSV-cp construct. A 1,100-bp NPTII fragment was used as probe; 10 μg of genomic DNA of these transgenic lines was digested with *Eco*RI. *MII, MIII* Digoxigenin (DIG)-labeled DNA markers

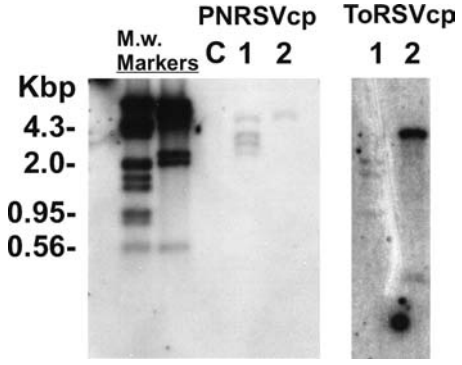

Fig. 4 DNA gel blots showing two lines containing the CP gene of PNRSV and two lines containing the CP gene of TomRSV. Blots were hybridized with the PCR-generated 633-bp PNRSVcp and 1700-bp TomRSV probes. *Lane C* contains a non-transformed control. DNA sizes indicated on the *left* were derived from the DIG-labeled DNA molecular-weight markers shown

evaluated by comparing the number of shoots produced at 7 weeks with the number of confirmed transgenic lines at the end of the experiment (approx. 28 weeks). These data (Table 3) show that a significant number of apparently untransformed escapes were produced under late selection when compared with early selection.

There was no statistical difference in the number of transgenic lines produced from *A. tumefaciens* strains EHA105 and LBA4404 but, in general, plasmid pGA482GG appeared to be more effective in introducing genes into plum hypocotyls, with more total transgenic lines produced in both early and late selection and also a higher transformation efficiency (Table 3).

Molecular analysis of kan-resistant shoots

PCR analysis indicated that all of the putative transformed shoots that were resistant to kan contained the NPTII gene (Fig. 2). DNA blotting of some of these shoots utilized a PCR-generated 1.1-kb NOS/NPTII probe and digestion with *Eco*RI. This restriction enzyme cuts once in the insert and distinguishes transgenic lines by producing unique banding patterns (Scorza et al. 1996). Transgenic lines with single and multicopy inserts were obtained (Fig. 3). The insertion of the PNRSV- and TomRSV-CP genes in selected lines was confirmed by DNA blotting (Fig. 4).

Rooting

Mante et al. (1991) reported that 20—25% of transgenic plum shoots rooted in a rooting medium containing 2.5 μM IBA and 75 mg l^{-1} kan. Scorza et al. (1994) increased rooting to 50% with a 2,500-μM IBA dip and the addition of 0.01 μM each of K and NAA to the medium. In the current study, we used the same basal medium (1/2-strength MS salts and vitamins), eliminated the IBA dip and evaluated kan and NAA levels in the medium. When the kan concentration was reduced by half—from 80 mg l^{-1} to 40 mg l^{-1}—the rooting percentage was only slightly higher (60%). However, when the NAA concentration of the standard medium was increased from 0.01 μM to 5 μM, the average percentage of rooting was 91%, without changing the kan concentration, which was 80 mg l^{-1}. The average number of roots per shoot was also somewhat, but not significantly, higher in this medium. However, the roots were significantly shorter at the higher NAA concentration (Table 4).

Table 4 Effect of a reduction in kanamycin (Kan) level from 80 mg l^{-1} to 40 mg l^{-1} and an increase in α-naphthaleneacetic acid (NAA) concentration from 0.1 μM to 5 μM on the rooting percentage and average root number and root length. Basal rooting medium consisted of 1/2-strength MS salts and vitamins, 0.1 μM K and 0.8% agar

Rooting treatment	Rooting[a] (%)	Root number[a]	Root length[a] (cm)
Kan 80/TIM 300 + NAA 0.1 μM	49b	2.7a	4.1b
Kan 40/TIM 300 + NAA 0.1 μM	60b	2.2a	4.4b
Kan 80/TIM 300 + NAA 5 μM	91a	4.3a	1.8a

[a] Means in columns with the same letters are not significantly different at $P<0.5$ according to the Student-Newman-Keuls test

Table 5 Effect of the acclimatization method (greenhouse or culture room) and size of the plantlets on the survival percentage and growth in height of transgenic plums after 2 weeks of acclimatization

Treatment	Size (cm)	Survival[a] (%)	Difference in height[a]
Greenhouse	1–1.5	70b	0.09b
	1.5–3	92a	0.34b
Culture room	1–1.5	100a	0.40b
	1.5–3	100a	2.02a

[a] Means in columns with the same letters are not significantly different at $P<0.5$ according to the Student-Newman-Keuls test

Acclimatization

Initial studies showed that the size of the rooted shoot rather than the number and length of the roots most dramatically affected plantlet survival in the greenhouse, and rooted shoots less than 1.0 cm in height did not survive (data not presented). Rooted shoots between 1 cm and 1.5 cm in height had a 70% rate of survival (Table 5), but if the this same-sized plantlet was transferred to sterile soil and placed in the culture room the survival percentage was 100%. Plantlets between 1.5 cm and 3 cm in height had a similarly high survival rate for both direct greenhouse and culture room-to-greenhouse acclimatization. When we measured the height of the plantlets before and after acclimatization, we observed that the plantlets placed in the culture room generally were taller than those in the greenhouse, but these differences were significant only for 1.5- to 3-cm-tall plantlets.

Discussion

Mante et al. (1991) regenerated and transformed shoots from plum hypocotyl slices after a 2-day co-cultivation with *Agrobacterium* followed by 2 weeks of culture on a medium containing antibiotic to control the growth of the *Agrobacterium* but without kan. After 2 weeks, the explants were transferred to a kan-containing medium. This is what we termed "late selection". Our results demonstrate that while regeneration rates for late selection were 48% versus 5.6% for early selection (approximately ninefold greater), ultimately, the number of confirmed transgenic lines was only 1.7-fold greater under late selection over all *Agrobacterium*/plasmid combinations. The high number of non-transformed shoots that developed prior to the initiation of kan selection required continual in vitro culture and selection with multiple transfers onto fresh selective medium. This process is costly in terms of time, chemicals and other consumables, and in terms of labor. Many other fruit tree species have been transformed using early selection. This strategy depends on the capability of a species and/or the tissue used for the regeneration/transformation to regenerate on a kan-containing medium. Some species are quite tolerant of kan. In citrus, for example, it has been suggested that regeneration in levels of kan as high as 100 mg l^{-1} is not a reliable indicator of transformation (Moore et al. 1992; Peña et al. 1995). Tolerance to kan or the protection of non-transformed cells from the selection agent by transformed cells (Cervera et al. 1998) may be responsible for the production of escapes. On the other hand, some species, such as apple, are highly sensitive to kan and selection levels can range from 2.5 mg l^{-1} to 16.0 mg l^{-1} for M26 (Norelli and Aldwinckle 1993). De Bondt et al. (1996) found that kan as a selective agent is toxic for apple cells and inhibits organogenesis. In pear, Merkulov et al. (1998) applied early selection with 50 mg l^{-1} kan. The kan-resistant callus was transferred to a regeneration medium with just 25 mg l^{-1} kan to allow for regeneration. Mante et al. (1991) found that non-transformed plum hypocotyl slices remained white and unexpanded and did not produce shoots when cultured on SRM in the presence of 10–75 mg l^{-1} kan. We used 80 mg l^{-1} kan to obtain transgenic shoots. Selection with a high kan concentration may force regeneration from specific transgenic cells, for example those with high NPTII expression due to insert location or transgene copy number. However, it may also reduce the total number of transformation events with certain vectors or transgenes, as seen in plasmid pGA482/GG-TomRSV-CP in which we obtained more transgenic lines with late selection. If specific interactions between selection pressure and the vector or gene of interest are not known or suspected, the decision to apply early versus late selection in plum depends on the expense of serial in vitro transfers onto selective medium versus the expense of re-isolating and culturing hypocotyl slices and on the effects of early versus late selection on the nature of the transformation events obtained. These effects might include differences in copy number and high versus moderate or low NPTII expression. It should be noted that effects of early versus late selection on copy number were not seen in the present study (data not presented).

Mante et al. (1991) reported that plum hypocotyl slices produced one to five putatively transformed shoots per slice. In Scorza et al. (1994), only one regenerated shoot was selected from each hypocotyl slice because in the case of multiple shoot production it was not clear whether these shoots were derived from the same or different transgenic events. In late selection, we also observed multiple shoot formation from the hypocotyl slices, but not in early selection, where only one shoot per slice was the common regeneration pattern. The results obtained with pGA482GGi/antisenseACO1 showed that only about one-third (3 of 8) of the shoots that formed from the same explant were from unique transformation events. Thus, in the case of late selection it appears that choosing one shoot per explant is warranted unless it is critical to maximize the number of transformants per explant, in which case each transgenic shoot must be assayed for uniqueness of insertion. These molecular assays add time and expense to a study.

Several studies have reported that strain EHA105 (or EHA101 from which it is derived) is the most effective for transformation in woody fruit species (De Bondt et al.

1994; Bell et al. 1999; Miguel and Oliveira 1999; Ainsley et al. 2001; Ghrobel et al. 1999). In plum, EHA101 has generally been used (Mante et al. 1991; Scorza et al. 1994). We did not note a difference between LBA44404 and EHA105 in our current work. However, the binary plasmid based on pGA482 (An et al. 1985) seems to be more efficient in introducing genes into plum than pBISN1. The transformation efficiency obtained ranged between 0.4% and 4.2%, which is higher than the range obtained by Scorza et al. (1994, 1995b), but still low compared with apple at 1.5–8.7% (Maximova et al. 1998) or citrus with up to 41% transformation efficiency (Cervera et al. 1998).

Putative transgenic shoots were rooted in 80 mg l^{-1} kan, the same concentration of kan used in the shoot regeneration and multiplication media. The rooting of non-transgenic shoots is inhibited at 30 mg l^{-1} kan. The high level of kan assured that the putative shoots that rooted were transgenic and potentially high NPTII expressers. Perhaps that was the reason that the reduction of kan to 40 mg l^{-1} in the rooting medium did not improve the rooting percentage. The increase of NAA to a level higher than that used previously (Scorza et al. 1994, 1995b) improved rooting. IBA has been used for the rooting of pear and apple (Maximova et al. 1998; Merkulov et al. 1998). NAA has been shown to produce too much callus in the rooting of some species, mainly when used at low concentrations for prolonged periods (Amin and Jaiswal 1987; Pierik 1988), but in plum, NAA did not produce excess callus on the roots although more abundant and shorter roots were produced. The use of NAA in the medium also allowed us to eliminate the pre-rooting IBA dip.

Prior work with plum acclimatization in our laboratory has followed the method of Mante et al. (1989) in which plantlets were transferred to the greenhouse once the adventitious roots were at least 2 cm long because it was felt that root length was important in the acclimatization process. However, in our current work, contrary to reports from investigations with apple (Van Telgan et al. 1992), we found that the length and number of the roots was not critically important. Using our improved rooting medium, we obtained an average of 4.3 roots per shoot, which may be above a threshold value where root number would be critical for acclimatization. We also observed that the length of the roots was not critical for acclimatization and that shoots with roots 0.5 cm long acclimatized well. We found shoot height to be critical. Since leaf number is positively correlated with shoot length, the number of leaves may be a determining factor for the successful greenhouse acclimatization of plum plantlets, as has been reported in hybrid walnut (*Junglans nigra* × *Juglans regia*) (Chenevard et al. 1995).

In summary, the rapid and efficient production of transgenic plum (*P. domestica*) plants can be optimized by using the basic methods described by Mante et al. (1991) and Scorza et al. (1995b), modified by the addition of 80 mg l^{-1} kan to the SRM immediately after co-cultivation (early selection), utilizing *A. tumefaciens* strains LBA4404, EHA101 or EHA105 with the plasmid pGA482. Transgenic shoots should be rooted in rooting medium containing 80 mg l^{-1} kan and 5 μM NAA. When shoots are at least 1.5 cm in height, they can be directly transferred to the greenhouse by using an anti-transpirant, covering the pots with an empty Magenta box and by placing the pots under 50% shade for 1 week. Using the protocols described in this report, we have consistently placed acclimated transgenic plum plants in the greenhouse within 6 months to 9 months after the initial co-cultivation of hypocotyl slices with *A. tumefaciens*.

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